

B. F. Ardelli · P. T. K. Woo

Therapeutic and prophylactic effects of isometamidium chloride (Samorin) against the hemoflagellate *Cryptobia salmositica* in chinook salmon (*Oncorhynchus tshawytscha*) and the effects of the drug on uninfected rainbow trout (*O. mykiss*)

Received: 1 March 2000 / Accepted: 18 July 2000

Abstract A series of compounds (triphenylmethanes, thiazines, xanthenes, benzidines, phenanthridiniums, naphthalamines, and diamidines) were screened for in vitro toxicity against *Cryptobia salmositica*. Isometamidium chloride (Samorin) was cryptobiacidal at low concentrations and was examined for therapeutic and prophylactic activities against *C. salmositica* in chinook salmon (*Oncorhynchus tshawytscha*). An intramuscular dose (1.0 mg/kg) of Samorin 3 weeks post-infection significantly reduced the parasitemia in adult chinook. A higher dose (2.5 mg/kg) eliminated the infection in 30% of adult fish and parasitemias were significantly reduced in the remaining infected fish. Juvenile chinook treated with 1.0 mg Samorin/kg at 2–3 weeks post-infection survived, while 100% of untreated control fish died from cryptobiosis. The high dose (2.5 mg/kg) was lethal to small fish (98.93 ± 12.09 g) and 50% died within 24 h of treatment, while all large fish (168.38 ± 13.87 g) survived. Samorin (1.0 mg/kg) did not affect growth, food consumption, complement, or hematocrit values in uninfected rainbow trout (*O. mykiss*).

normally occurs through the bite of a blood-sucking leech, *Piscicola salmositica* (Becker and Katz 1965). In Canada, the parasite is found in the Fraser River system, tributaries adjacent to the Fraser River, and in rivers on both the east and west coasts of Vancouver Island (Bower and Margolis 1984).

Clinical signs of salmonid cryptobiosis in experimentally infected rainbow trout (*O. mykiss*) include exophthalmia, splenomegaly, general edema, abdominal distention with ascites fluid, and both microcytic and hypochromic anemia (Woo 1979). The parasite secretes hemolysins; and red blood cells in infected animals become anti-globulin positive. In addition, the pathogen causes anorexia and immunodepression in its host (Wehnert and Woo 1981; Jones et al. 1986; Thomas and Woo 1988, 1989a, b; Li and Woo 1991). The histopathology appears to be a generalized inflammatory reaction both within connective tissues and in the reticulo-endothelial system (Bahmanrokh and Woo 1994). In addition there are focal hemorrhages, congestion of blood vessels, occlusion of capillaries by parasites, and edema within kidney glomeruli (Putz 1972).

In the Soleduck Hatchery (Washington State, USA) about 50–60% of spring chinook salmon broodstock annually die from cryptobiosis (L. Peck, personal communication). In Canada, an outbreak of the disease occurred in 1997 and large numbers of chinook salmon smolts and broodstock died in a hatchery on Vancouver Island (S. Sakisida, personal communication). Although *C. salmositica* is recognized as a lethal pathogen in both semi-natural and intensive salmon culture facilities on the Pacific coast of North America (Bower and Thompson 1987), there is no chemotherapy.

There is a live vaccine, an attenuated strain of *C. salmositica*, that protects fish from cryptobiosis (Woo and Li 1990; Ardelli et al. 1994; Sitja-Bobadilla and Woo 1994; Li and Woo 1995). Vaccinated fish are protected for at least 2 years (Li and Woo 1995) and the efficacy of the vaccine is not reduced when vaccinated fish are transferred from freshwater to seawater (Li and Woo 1995). To control cryptobiosis, the strategy is to

Introduction

Cryptobia salmositica is a pathogenic hemoflagellate of the economically important *Oncorhynchus* spp on the west coast of North America (Woo and Poynton 1995). The parasite causes disease and mortality in all *Oncorhynchus* spp (Woo 1992, 1994) and transmission

P. T. K. Woo (✉)
Department of Zoology and Axelrod Institute of Ichthyology,
University of Guelph, Guelph, Ontario, N1G 2W1, Canada
e-mail: pwoo@uoguelph.ca
Tel.: +1-519-8244120; Fax: +1-519-7671656

B. F. Ardelli · P. T. K. Woo
Department of Zoology, College of Biological Science,
University of Guelph, Guelph, Ontario, N1G 2W1, Canada

vaccinate salmon pre-smolts to protect them while in freshwater and when they return from the sea as adults (Woo 1992; Li and Woo 1995). The vaccine is not therapeutic and thus chemotherapy is necessary for unvaccinated broodstock that become infected with *C. salmositica* on return to freshwater. Presently, the practice in some west coast hatcheries is to collect broodstocks as they migrate to their spawning grounds. These infected fish are held for several weeks before they are stripped of eggs and sperm and mortality may occur if they are heavily infected.

The objectives of this study were to: (1) screen potential drugs in vitro for use in chemotherapy, (2) examine the therapeutic and prophylactic effects of the candidate drug against *C. salmositica* in chinook salmon (*O. tshawytscha*), and (3) examine the effects of the drug in uninfected rainbow trout (*O. mykiss*).

Materials and methods

In vitro culture of *C. salmositica*

A cloned strain of *C. salmositica* was used to infect rainbow trout held at 10 °C. The strain was initially isolated from *Piscicola salmositica* and details of the cloning of the parasite have been described (Woo 1979). The pathogenicity and infectivity of *C. salmositica* was maintained by serial syringe passage in rainbow trout. To culture the parasite, blood from an infected trout was withdrawn and inoculated aseptically into sterile flasks containing minimum essential medium (MEM) supplemented with Hank's salts, L-glutamine, 25 mM Hepes buffer, and 25% heat-inactivated fetal bovine serum (Woo and Li 1990). *C. salmositica* was not maintained in MEM for more than 8 weeks, to ensure its pathogenicity to trout (Woo and Thomas 1991).

Maintenance of fish

Chinook salmon fry were obtained from the Ringwood Hatchery, Ontario Ministry of Natural Resources, Canada. The chinook salmon are the progeny of a fifth generation of salmon introduced into Lake Ontario. The stock is of mixed parentage which originated from the west coast. Rainbow trout were obtained from Silvercreek Aquaculture (Erin, Ontario, Canada). All fish were maintained in 125 l tanks with equatorial photoperiod in aerated recirculating well water (10 °C). Fish were fed a 52% protein diet (Martin Feed Mills, Elmira, Ontario, Canada) daily and food was provided ad libitum.

In vitro assay for cryptobiacidal drug(s)

A sample (25 µl) of chilled cold-blooded vertebrate Ringer's solution (CBVR, pH 7.3) was added to each of 24 wells in a 96-well microtiter plate kept on ice. To each well ($n = 24$) was added 1,500 *C. salmositica* from cultures (washed three times in CBVR) in 25 µl of CBVR, followed by 25 µl of different concentrations (0.1 µM, 1.0 µM, 10 µM, 100 µM, or 1,000 µM) of each of the following chemicals: crystal violet, malachite green, pararosaniline, acridine orange, pyronin Y, toluidine blue, methylene blue, trypan blue, congo red, diminiazene acetate (Berenil; Hoescht, Germany), isometamidium chloride (Samorin; Rhône Mérieux, France), quinapyramine sulfate (Antrycide; Bayer, UK), and a sulfonated naphthylamine dye (Suramin; Bayer, UK). All compounds were dissolved in sterile phosphate-buffered saline (pH 7.3). Plates were incubated for 3 h at 11 °C, after which the wells were examined for live parasites using an inverted microscope (ocular 10× and

objective 10×). The endpoint was the concentration of drug which did not contain living parasites.

Antigen-capture enzyme-linked immunosorbent assay to detect isometamidium in fish plasma

The wells of a 96-well microtiter plate were coated with 50 µl of a 1:1,000 dilution of purified anti-isometamidium antibodies (produced in trout) in carbonate-bicarbonate coating buffer, pH 7.3. Plates were placed at 37 °C for 2 h and then washed twice in Tris-buffered saline (TBS, pH 7.5) and Tween-Tris buffered saline (TTBS, pH 7.5). All subsequent washings were performed in this manner. Vacant sites were blocked with 200 µl of 5% skimmed milk in TBS for 1 h at 37 °C, then washed and 50 µl of test plasma was added to each well before being incubated at 37 °C for 2 h. After washing, 50 µl of a 1:1,000 dilution of anti-isometamidium antibodies were added to each well and incubated at 37 °C for 2 h. Plates were washed and then a 1:1,000 dilution of affinity-purified peroxidase-labeled goat anti-trout immunoglobulin (Kirkegaard and Perry, Mississauga, Ontario, Canada) was added to each well. Plates were incubated (37 °C for 1 h), washed and then 50 µl of substrate {2,2'-azino-di-[3-ethyl-benzthiazoline sulfonate (6)]} was added. Plates were shaken for 1 min and the absorbance was read at 405 nm using a microplate reader. Negative controls were wells containing no anti-isometamidium antibodies, plasma without isometamidium, and no goat anti-trout immunoglobulin.

Therapeutic and prophylactic effects of isometamidium chloride on *C. salmositica* in adult *O. tshawytscha*

Experiment 1

Thirty chinook salmon were divided into three groups ($n = 10$ /group): group A (200.80 ± 52.64 g), group B (187.44 ± 43.51 g), and group C (230.12 ± 71.32 g). Each fish in groups A and B was inoculated intraperitoneally (i.p.) with 5,000 *C. salmositica* and fish in group C were inoculated intramuscularly (i.m.) with 1.0 mg isometamidium/kg. At 3 weeks post-infection, fish in group B were inoculated i.m. with 1.0 mg isometamidium/kg. Five weeks after the start of the study, fish in group C were inoculated i.p. with 5,000 *C. salmositica*. Blood samples (0.1 ml/fish) were obtained prior to infection and then weekly for 10 weeks post-infection. Parasitemias were determined using a hemocytometer (when infections were high) or the hematocrit centrifuge technique (very sensitive and used for low infections; Woo 1969). Anemia was used as an indicator of disease and was measured by determining the packed cell volume (PCV). Concentrations of isometamidium were determined using an enzyme-linked immunosorbent assay (ELISA; Ardelli and Woo 2000).

Experiment 2

Thirty chinook salmon were divided into three groups ($n = 10$ /group): group D (292.08 ± 39.64 g), group E (240.82 ± 21.60 g), and group F (245.55 ± 25.07 g). Treatment of experimental and control groups was as described for experiment 1, except that the group which received isometamidium prophylactically (group F) was infected at 3 weeks after injection as opposed to 5 weeks (see experiment 1). Parasitemias, PCV, and concentrations of isometamidium in plasma were determined.

Experiment 3

Forty chinook salmon were divided into two groups ($n = 20$ /group): group G (98.93 ± 12.09 g) and group H (168.38 ± 13.87 g). Each fish in groups G and H was inoculated i.p. with 5,000 pathogenic *C. salmositica*. At 3 weeks after the start of the experiment, fish in groups G and H were randomly divided into subgroups ($n = 10$ /subgroup): G1 (97.76 ± 13.34 g), G2

(100.10 ± 10.84 g), H1 (170.45 ± 11.68 g), and H2 (166.32 ± 16.06 g). Fish in groups G2 and H2 were inoculated i.m. with 2.5 mg isometamidium chloride/kg. Parasitemias, PCV, and concentrations of isometamidium in plasma were determined weekly.

Therapeutic and prophylactic effects of isometamidium chloride on *C. salmositica* in juvenile *O. tshawytscha*

Experiment 1

Forty-five juvenile chinook salmon were divided into three groups ($n = 15/\text{group}$): group I (32.36 ± 3.25 g), group J (34.69 ± 2.43 g), and group K (32.53 ± 2.69 g). Each fish was inoculated i.p. with 5,000 *C. salmositica*. At 2 weeks post-infection, fish in group I were inoculated i.m. with 1.0 mg isometamidium/kg and fish in group J were treated with a similar dose at 3 weeks post-infection. Fish were not sampled but monitored daily for mortality.

Experiment 2

One hundred and twenty juvenile chinook salmon were divided into three groups ($n = 40/\text{group}$): group L (1.0 mg/kg at 2 weeks, 66.75 ± 5.76 g), group M (1.0 mg/kg at 3 weeks, 67.32 ± 4.38 g), and group N (untreated infected controls, 65.28 ± 3.97 g) and were then further divided into subgroups ($n = 20/\text{subgroup}$): L1 (69.96 ± 3.74 g), L2 (63.53 ± 6.15 g), M1 (67.85 ± 4.99 g), M2 (66.79 ± 4.35 g), N1 (69.08 ± 3.98 g), and N2 (61.48 ± 4.92 g). Experimental and control groups were treated as described in experiment 1. Fish in subgroups L1, M1, and N1 were bled weekly and parasitemias, PCV, and concentrations of isometamidium in plasma were determined. Subgroups L2, M2, and N2 were not sampled but were monitored daily for mortality.

Effects of isometamidium on complement activity in uninfected rainbow trout

Twenty rainbow trout were randomly divided into two groups: group O (209.06 ± 9.133 g) and group P (224.17 ± 16.827 g). Blood samples (0.2 ml/fish) were obtained weekly from each fish in each group for 2 weeks. After 2 weeks, fish in Group O were inoculated i.m. with 0.2 ml of saline and fish in Group P were inoculated i.m. with 1.0 mg isometamidium chloride/kg. Blood samples were obtained weekly for 3 weeks. Plasma samples were separated by centrifugation and kept on ice. The hemolytic activity of complement was determined immediately as follows.

Plasma samples from fish were divided into two equal portions. One portion was heat-inactivated at 37 °C for 30 min and the other portion was kept on ice. A 1- μl aliquot of a 10% suspension of rabbit red blood cells (RRBC), washed three times in saline, was added to 0.5 ml of either fresh or heat-inactivated rainbow trout plasma. The suspensions were incubated at 20 °C for 45 min. After incubation, hemolysis of red blood cells was measured by calculating the amount of hemoglobin released. Suspensions were centrifuged for 1 min at 14,000 g to pellet the RRBC. An aliquot of 60 μl of supernatant was removed and placed in a 1.5-ml test tube. To the tube was added 940 μl of Drabkin's reagent (Sigma). The sample was vortexed for 10 s and allowed to stand for 15 min. The absorbance of the sample was recorded at 540 nm against a reagent blank. Hemoglobin was calculated from a standard curve. The amount of hemoglobin released was proportional to the amount of complement present (Thomas and Woo 1989b).

Effects of isometamidium on PCV, weight gain, and food consumption in uninfected rainbow trout

Forty disease-free rainbow trout were randomly divided into two groups ($n = 20/\text{group}$): group Q (235.62 ± 33.25 g) and group R (227.71 ± 31.17 g) and were maintained at 10 °C. The average weights in the two groups were not significantly different. Fish were

fed 5GR trout pellets, at the same time each day ad libitum, until food consumption between the two groups was not significantly different. The amount of food consumed was recorded daily. At 4 weeks after the start of the experiment, fish in group Q were given an i.m. injection of saline (0.2 ml) and fish in group R were inoculated i.m. with 1.0 mg isometamidium/kg. Food consumption, weight gain, and PCV were monitored before and for 5 weeks after drug injection.

Statistical analysis

Data were analyzed using a one-way analysis of variance (ANOVA). When the distribution was not normal and the variances were unequal, an ANOVA on ranks was used. Significantly different results were analyzed using a pairwise multiple comparison procedure. A Student–Newman–Keuls test was used for equal sample sizes and a Dunn's test for unequal sample sizes. Significance was evaluated at $P \geq 0.05$.

Results

In vitro toxicity assay for cryptobiacidal drugs

In the triphenylmethane structural class, crystal violet and malachite green were cryptobiacidal, while pararosaniline had no visible effects against *C. salmositica*. Crystal violet was lethal at low concentrations (0.1 μM) while malachite green was lethal at higher concentrations (10 μM). Also, malachite green exhibited some activity at 1 μM . The xanthene structural class also exhibited some cryptobiacidal activity. Acridine orange lyzed *C. salmositica* at low concentrations (0.1 μM), while pyronin Y exhibited cryptobiacidal activity at higher concentrations (10 μM). Both thiazine dyes (toluidine blue and methylene blue) lyzed *C. salmositica*, but only at high concentrations (10.0 μM). Methylene blue showed some activity at 1.0 μM . Within the benzidine-azo structural class, congo red was cryptobiacidal at high concentrations (1,000 μM). In contrast, trypan blue did not lyze *C. salmositica*. With the exception of isometamidium chloride, none of the curative trypanocidal drugs were cryptobiacidal. Isometamidium chloride lyzed *C. salmositica* at low concentrations (0.1 μM ; Table 1).

Therapeutic and prophylactic effects of isometamidium in adult *O. tshawytscha*

Experiment 1

Parasitemias in fish (group B – therapeutic) treated with 1.0 mg isometamidium/kg 3 weeks post-infection were significantly lower than those in infected controls (group A) at 2 weeks ($P = 0.0077$) and 3 weeks ($P = 0.0567$) after treatment (or 5 weeks and 6 weeks post-infection). Parasitemias remained lower in group B, but were not significantly different from the controls at 4 weeks and 5 weeks after treatment (or 7 weeks and 8 weeks post-infection; Fig. 1). By 8 weeks post-infection there was

Table 1 Summary of compounds tested in vitro for toxic effects against *Cryptobia salmositica*. Values given are (number of replicates with living *C. salmositica*)/(total number of replicates)

Compound	Structural class	0.1 μ M	1.0 μ M	10 μ M	100 μ M	1,000 μ M
Crystal violet	Triphenylmethane	0/24	0/24	0/24	0/24	0/24
Malachite green	Triphenylmethane	24/24	22/24	0/24	0/24	0/24
Pararosaniline	Triphenylmethane	24/24	24/24	24/24	24/24	24/24
Acridine orange	Xanthene	0/24	0/24	0/24	0/24	0/24
Pyronin Y	Xanthene	24/24	24/24	0/24	0/24	0/24
Toluidine blue	Thiazine	24/24	16/24	0/24	0/24	0/24
Methylene blue	Thiazine	24/24	24/24	0/24	0/24	0/24
Trypan blue	Benzidine azo	24/24	24/24	24/24	24/24	24/24
Congo red	Benzidine azo	24/24	24/24	24/24	24/24	0/24
Suramin	Naphthylamine	24/24	24/24	24/24	24/24	24/24
Diminiazene aceturate	Diamidine	24/24	24/24	24/24	24/24	24/24
Antricyde chloride	Phenanthridinium	24/24	24/24	24/24	24/24	24/24
Isometamidium chloride	Phenanthridinium	0/24	0/24	0/24	0/24	0/24

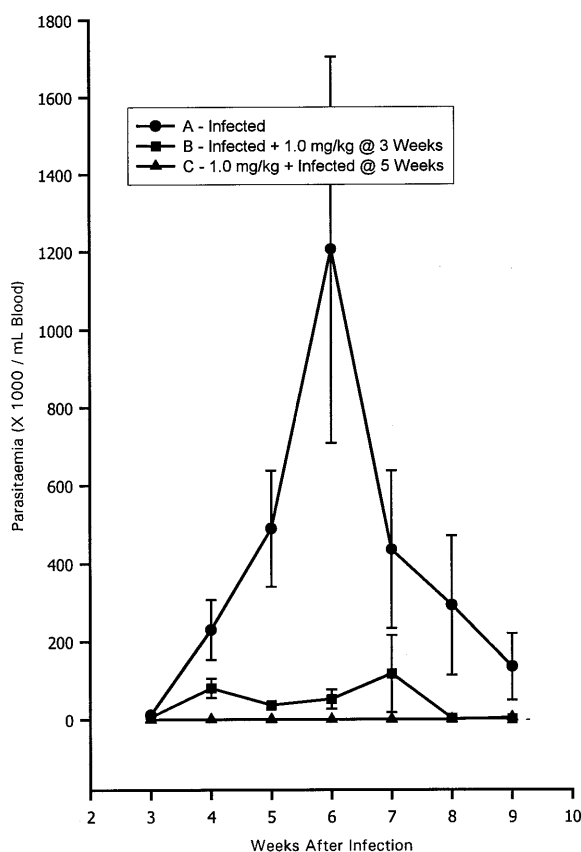


Fig. 1 Parasitaemia in adult chinook salmon, *Oncorhynchus tshawytscha*, inoculated with 5,000 *Cryptobia salmositica* (experiment 1) and treated therapeutically (3 weeks after infection) or prophylactically (infected 5 weeks after treatment) with 1.0 mg isometamidium chloride/kg

50% mortality in infected untreated controls (group A) and 30% mortality in fish treated with 1.0 mg isometamidium/kg (group B).

At 1 week after infection (5 weeks after drug inoculation), 50% of fish injected with isometamidium (Group C – prophylactic) had detectable infections. By 4 weeks post-infection (8 weeks after drug inoculation), all fish in group C had a detectable infection, although

infections were only detectable using the hematocrit centrifuge technique. In contrast, the infected untreated control fish had high parasitemias at 4 weeks post-infection (Fig. 1).

The anemia was more severe in non-treated fish in comparison to treated fish. The differences were significant between infected controls (group A) and fish in group B (therapeutic – treated with 1.0 mg isometamidium/kg at 3 weeks) at 3 weeks ($P = 0.0255$), 4 weeks ($P = 0.0547$), 6 weeks ($P = 0.0002$), 7 weeks ($P = 0.0003$), and 8 weeks ($P = 0.0247$) after infection. PCV in group C (prophylactic) was significantly higher than PCV in infected controls (group A) at 6 weeks ($P = 0.0005$), 7 weeks ($P = 0.0015$), and 8 weeks ($P = 0.0033$) after infection.

The concentration of isometamidium was highest in plasma at 2 weeks after treatment and then declined. In group C (prophylactic), drug levels peaked 2 weeks after injection (0.019 ± 0.0028 mg/ml) and were still detectable after 7 weeks (0.0009 ± 0.0097 mg/ml). Similarly, in group B (therapeutic), the concentration of isometamidium peaked 2 weeks after treatment (0.0219 ± 0.0012 mg/ml) and then declined, but was still detectable after 5 weeks (0.0015 ± 0.0002 mg/ml). In both groups, isometamidium was detectable within 1 week of administration.

Experiment 2

Another experiment, similar to experiment 1, was performed to confirm the therapeutic and prophylactic effects of isometamidium chloride in adult chinook salmon. This experiment was different from experiment 1 in that fish were infected (group F – prophylactic) at 3 weeks, as opposed to 5 weeks (experiment 1), after inoculation of the drug. Significant differences in parasitemias were not detected between groups D (infected controls), E (therapeutic), and F (prophylactic) at 1–4 weeks after infection. Parasitemias were higher in infected controls (group D), in comparison to fish treated with isometamidium. In group E (therapeutic), parasitemias declined after treatment and remained low.

At 4 weeks after drug injection (1 week after infection), fish in group F (prophylactic) were not infected. However, 4/10 fish (5 weeks after drug injection), 6/10 fish (6 weeks after drug injection), and 7/10 fish (7 weeks after drug injection) later developed low infections (Fig. 2). There was only 10% mortality in fish treated with 1.0 mg isometamidium/kg at 3 weeks post-infection (group E – therapeutic).

Similar to experiment 1, the anemia was more severe in non-treated chinook, in comparison to treated fish. Also, concentrations of isometamidium were detected at 1 week after treatment, peaked in treated groups 2 weeks after treatment, and then declined. Also, isometamidium was still detectable in group E (therapeutic) and group F (prophylactic) 8 weeks post-infection.

Experiment 3

Parasitemias were not significantly different between groups G and H before treatment. After treatment, significant differences were detected between subgroup G1 (infected controls) and fish treated with 2.5 mg

isometamidium/kg at 2 weeks (G2; $P = 0.0162$) and 3 weeks ($P = 0.0311$) after treatment (4 weeks and 5 weeks after infection). Significant differences in parasitemias were detected between subgroup H1 (infected controls) and subgroup H2 (treated at 3 weeks with 2.5 mg/kg) at 2 weeks ($P = 0.0123$) after treatment (5 weeks after infection; Fig. 3). Furthermore at 24 h post-treatment, there was 50% mortality in smaller fish treated with 2.5 mg isometamidium/kg; all larger fish survived the dose. By 7 weeks post-infection (end of study) there was 100% (group G1) and 60% (group H1) mortality in untreated infected controls. In subgroup H2 (larger fish treated with 2.5 mg/kg) all fish survived, while only 50% of fish in subgroup G2 (smaller fish treated with 2.5 mg/kg) survived.

Similar to the previous experiments, PCV declined in non-treated infected controls and was lower than that in treated groups. Concentrations of isometamidium were highest 2 weeks after treatment (5 weeks after infection). The drug concentration was highest in the plasma of smaller fish at 2 weeks after treatment (0.052 ± 0.0029) and was approximately twice that of larger fish (0.0245 ± 0.0056).

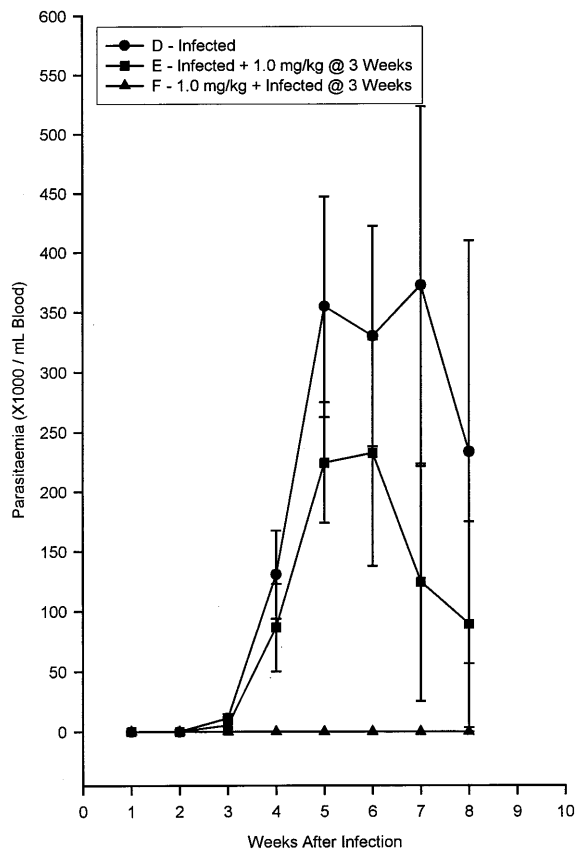


Fig. 2 Parasitemia in adult chinook salmon, *O. tshawytscha*, inoculated with 5,000 *C. salmositica* (experiment 2) and treated therapeutically (3 weeks after infection) or prophylactically (infected 3 weeks after treatment) with 1.0 mg isometamidium chloride/kg

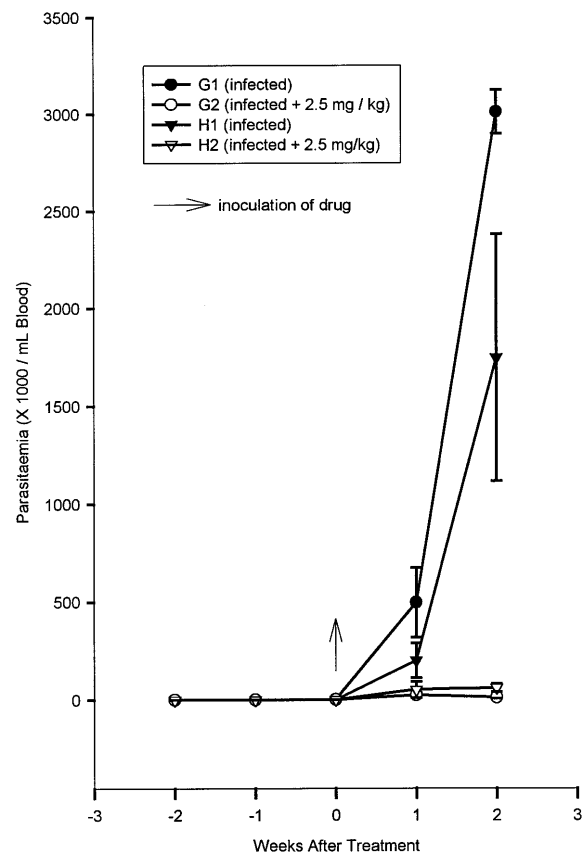


Fig. 3 Parasitemia in small and large chinook salmon, *O. tshawytscha*, infected with 5,000 *C. salmositica* and treated with 2.5 mg isometamidium chloride/kg

Therapeutic and prophylactic effects of isometamidium in juvenile *O. tshawytscha*

Experiment 1

All 15 infected untreated juvenile chinook salmon (group I) died from the disease within 8 weeks – 8, 3, 3, and 1 fish died at 5, 6, 7, and 8 weeks post-infection, respectively. In contrast, mortality was very low in fish treated at 2 weeks (group J) and 3 weeks (group K) after infection. Only one fish in each group died; and the remaining 14 fish (93%) in each group survived the disease.

Experiment 2

This experiment was to confirm that isometamidium was therapeutic in juvenile chinook salmon. All treated juvenile chinook salmon survived (groups M1, M2, N1, and N2), while 100% of controls (groups L1 and L2) died from cryptobiosis. In control groups, the mortality was 50% at 6–7 weeks after infection. Parasitemias in infected controls (group L1) were significantly higher than treated fish at 2 weeks (group M1; $=0.0028$) after treatment (Fig. 4).

PCV in infected controls (group L1) was lower than in treated fish (groups M1 and N1) and was most severe (average of 5%) at 6 weeks post-infection. Isometamidium was highest in treated fish at 2 weeks after injection and was still detectable at 7–8 weeks post-treatment.

Effects of isometamidium on fish biology

Isometamidium, at a dose of 1.0 mg/kg, did not affect complement activity, PCV, weight gain, or food consumption in uninfected rainbow trout. Complement activity was not significantly different between fish in group O (injected with saline) and group P (injected with 1.0 mg isometamidium/kg). Injection of uninfected rainbow trout with 1.0 mg isometamidium chloride/kg did not cause anemia. The PCV in both groups fluctuated but was not significantly different.

The weights of rainbow trout after treatment with isometamidium chloride did not differ significantly from untreated controls (group P). Fish in both groups gained an average of 0.2 kg over the 5 week study period. Although food consumption decreased in both groups at 1 week after treatment (either with saline or isometamidium), the amount of food consumed overall was not significantly different.

Discussion

The *in vitro* study suggested that crystal violet might be a potential therapeutic agent against salmonid cryptobiosis (present study). Low concentrations of the dye

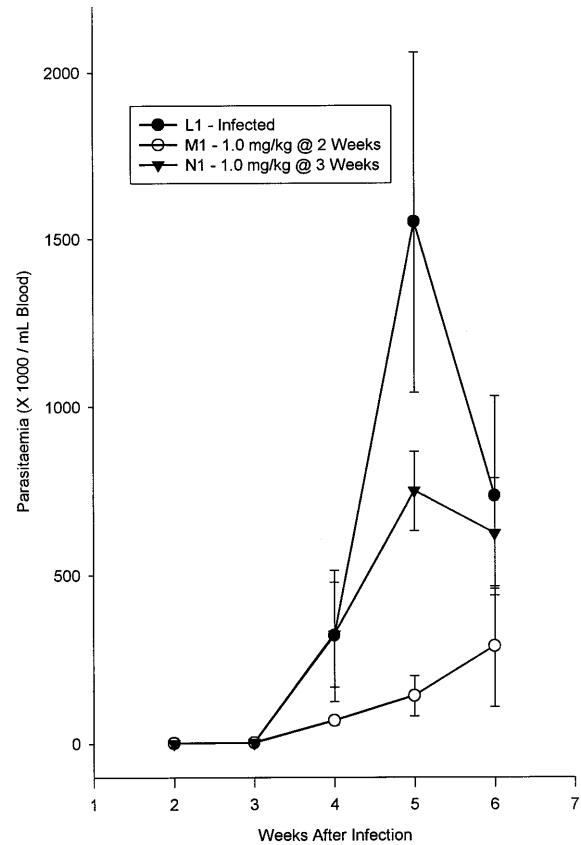


Fig. 4 Parasitemias in juvenile chinook salmon, *O. tshawytscha*, treated with 1.0 mg isometamidium chloride/kg at 2 weeks or 3 weeks after infection with 5,000 *C. salmositica* (experiment 2)

inhibited multiplication of *C. salmositica*, altered the infectivity of the parasite, and caused lesions on mitochondrial and nuclear membranes. An earlier study indicated that at least 200 mM of crystal violet was required to lyse all *C. salmositica* in blood incubated under *in vitro* conditions (Ardelli and Woo 1998). However, an *in vivo* study demonstrated that 200 mM of crystal violet delivered *i.p.* resulted in 74% mortality in juvenile rainbow trout (unpublished observations). Thus, crystal violet would not be a useful therapeutic agent against *C. salmositica* unless its toxicity to fish could be reduced.

Similar to crystal violet, isometamidium chloride also lysed *C. salmositica* at low concentrations (0.1 μ M) under *in vitro* conditions (present study). This drug is widely used to control trypanosomiasis in cattle, sheep, goats, buffalos, donkeys, horses, camels, and dogs (Kinabo et al. 1989) and is currently the only drug available for the prophylaxis of bovine trypanosomiasis (Kinabo and Bogan 1987). Since there is no effective drug against salmonid cryptobiosis (Woo and Poynton 1995) and there are many similarities between piscine cryptobiosis and bovine trypanosomiasis, isometamidium may have similar effects against *C. salmositica*.

Some of the triphenylmethane dyes lysed *C. salmositica* at low concentrations under *in vitro* conditions

(present study). This is similar to the pathogenic trypanosomes, as some of these dyes have trypanocidal activity (Williamson 1970). Crystal violet is the only triphenylmethane dye which is currently used as a trypanocide and is routinely added to blood in many South American blood banks to prevent transmission of *Trypanosoma cruzi* (Docampo and Moreno 1990). The xanthene series of compounds also lyzed *C. salmositica* under in vitro conditions. Although there are no reports of trypanocidal activity by xanthenes, several structural analogs within this series have anti-malarial properties. The azo dyes are trypanocidal, but have only a weak cryptobiocidal activity (present study).

Isometamidium chloride was the only trypanocidal drug tested which lyzed *C. salmositica* under in vitro conditions. Antrycide and isometamidium are both phenanthridinium compounds, yet show differences in their activity against *C. salmositica*. Isometamidium was produced by linking a portion of Berenil to ethidium, yet Berenil does not have effects against *C. salmositica* under in vitro conditions. Structurally, Berenil is related to trypan blue and similarly does not show cryptobiocidal activity. Suramin was the only trypanocide tested which is routinely used against both human trypanosomiasis (*T. b. gambiense* and *T. b. rhodesiense*) and animal trypanosomiasis (*T. b. brucei* and *T. evansi*). Suramin has no activity against *T. vivax* or *T. congolense* infections of cattle; and it only has a therapeutic effect on *T. simiae* in pigs. Its main use against animal trypanosomiasis has been for *T. evansi* infections of camels (Williamson 1970). *C. salmositica* and *Trypanosoma* spp are phylogenetically related, yet show differences in their susceptibilities to commonly used trypanocides (present study). Thomas and Woo (1991) demonstrated that antimicrobial agents (combination of penicillin, streptomycin, and amphotericin B), which do not affect the viability of trypanosomes in vitro, had significant effects against *C. salmositica* from the blood of infected trout and from cultures.

Chinook salmon treated intramuscularly at 3 weeks post-infection with 1.0 mg/kg had significantly lower parasitemias than infected controls. The manufacturer recommends isometamidium be delivered intramuscularly at doses of 0.25–1.0 mg/kg body weight for therapeutic and prophylactic uses (Sutherland et al. 1992). Isometamidium had prophylactic and therapeutic activities in chinook salmon at 1.0 mg/kg, eliminating the infection in some fish and lowering the parasitemia in others. A higher dose (2.5 mg/kg) was effective in preventing infection for 7 weeks (unpublished), but was toxic and 70% of fish died. Cholinergic fibers secrete acetylcholine; and cholinergic receptor sites include motor fibers to striated muscle and fibers to sympathetic ganglia from the central nervous system. Apparently these receptor sites are occupied by isometamidium when concentrations in the blood are high. When the drug level decreases, the binding of the drug to the receptors is reversed and recovery from acute reactions quickly ensues (Philips et al. 1967). This may occur in

chinook salmon which survived a high dose of isometamidium (2.5 mg/kg). Similar observations were made in camels treated intravenously with 0.5 mg or 1.0 mg isometamidium/kg (Ali and Hassan 1986).

The high dose was more toxic to small fish than to large fish (of the same age and stock) and it eliminated the infection in 30% of treated fish. In addition, 50% of small fish died within 24 h of treatment (2.5 mg/kg), while all large fish survived the injection. Isometamidium remains at the site of intramuscular injection and is slowly released to the plasma (Murilla et al. 1996). Smaller fish have less muscle and isometamidium may be released more quickly from these sites. Thus, the concentration of isometamidium would be much higher in plasma, bind to more cholinergic receptor sites, and result in the more toxic effects observed in small fish. This was confirmed using the ELISA (Ardelli and Woo 2000), which showed that concentrations of isometamidium in the plasma of smaller fish was higher than that in larger fish at 2 weeks after treatment (present study).

Isometamidium is the most widely used chemoprophylactic agent against trypanosomiasis (Whitelaw et al. 1986). The duration of prophylaxis is variable and can last 14–36 weeks (Robson 1962; Fairclough 1963; Kirby 1964; Weisenhütter et al. 1968). Chinook salmon inoculated with 1.0 mg isometamidium chloride/kg had better prophylactic protection when challenged at 3 weeks than at 5 weeks. This was because the concentration of isometamidium in plasma was higher at 3 weeks. In the present study, it took 2 weeks before the therapeutic effects of isometamidium were observed in both juvenile and adult chinook salmon. This was because of the slow release of the drug into the plasma; in *Oncorhynchus* spp, the concentrations of drug were highest at 2 weeks post-treatment and this coincided with a significant decrease in parasitemia. In mammals, isometamidium declined rapidly in the plasma within the first 24 h after i.m. injection (Murilla et al. 1996). The much slower release of the drug in *O. tshawytscha* might be because our fish were held at 10 °C. The metabolic process of fish is low (poikilothermic animal) and the heart muscles and associated chambers do not pump blood as reliably or as strongly as do the hearts of more advanced vertebrates (e.g. birds and mammals). The muscle tissue of fish is poorly vascularized, making diffusion into the system slow. All of these may account for the slow release of isometamidium in chinook salmon.

There are studies on the acute toxicity of isometamidium, but little is known on the chronic effects of the drug (Philips et al. 1967; Ali and Hassan 1986; Kinabo et al. 1991; Gimbi and Kinabo 1992). A single i.m. dose of 0.6 mg isometamidium/kg is lethal to rats. There is up to 10% loss in body weight in the first 4 days after i.p. injection, with some mortality (11–36%) when it is injected subcutaneously (Philips et al. 1967). Other studies indicate that isometamidium produces an anemia in rabbits (Ali and Haroun 1984). Camels treated intravenously with 0.5 mg/kg or 1.0 mg/kg showed lacrimation, salivation, trembling, restlessness, frequent

urination, and defecation, followed by diarrhea (Ali and Hassan 1986). In the present study, isometamidium at a dose of 1.0 mg/kg did not affect growth, food consumption, or hematocrit values in rainbow trout. These are important factors to consider in aquaculture; and future work should examine the chronic toxicological effects of isometamidium.

The present study demonstrated that isometamidium chloride was effective against salmonid cryptobiosis. A dose of 1.0 mg/kg significantly lowered the parasitemia in fish and also prevented mortality in juvenile chinook salmon. This study is part of an ongoing project on the development of protective strategies against salmonid cryptobiosis.

Acknowledgements This study was supported by grants from the Natural Sciences and Engineering Research Council (Canada) to P.T.K. Woo. All research was conducted at the Aquatic Sciences Facility (Hagen Aqualab) at the University of Guelph. The authors gratefully acknowledge Dr. A. S. Peregrine (Ontario Veterinary College, Department of Pathobiology) for donating the isometamidium chloride used in this study.

References

- Ali BH, Haroun EM (1984) Acute toxicity of samorin (isometamidium chloride) in rabbits. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* 78: 419–423
- Ali BH, Hassan T (1986) Some observations on the toxicosis of isometamidium chloride (Samorin) in camels. *Vet Hum Toxicol* 28: 424–426
- Ardelli BF, Woo PTK (1998) The in vitro effects of crystal violet on the pathogenic piscine hemoflagellate *Cryptobia salmositica* Katz, 1951 (Sarcocystidophora: Kinetoplastida). *Parasite* 5: 27–36
- Ardelli BF, Woo PTK (2000) An antigen-capture enzyme-linked immunosorbent assay (ELISA) to detect isometamidium chloride in *Oncorhynchus* spp. *Dis Aquat Org* 37: 195–203
- Ardelli BF, Forward GM, Woo PTK (1994) Brook charr, *Salvelinus fontinalis*, and cryptobiosis: a potential salmonid reservoir host for *Cryptobia salmositica* Katz, 1951. *J Fish Dis* 17: 567–577
- Bahmanrokh M, Woo PTK (1994) The histopathology of cryptobiosis in juvenile *Oncorhynchus mykiss* (Walbaum). 8th Int Cong Parasitol 2: 434 (Abstract)
- Becker CD, Katz M (1965) Infections of the hemoflagellate *Cryptobia salmositica* Katz 1951, in freshwater teleosts of the Pacific coast. *Trans Am Fish Soc* 94: 327–333
- Bower SM, Margolis L (1984) Detection of infection and susceptibility of different Pacific salmon stocks (*Oncorhynchus* spp) to the haemoflagellate *Cryptobia salmositica*. *J Parasitol* 70: 273–278
- Bower SM, Thompson AB (1987) Hatching of the Pacific salmon leech (*Piscicola salmositica*) from cocoons exposed to various treatments. *Aquaculture* 66: 1–8
- Docampo R, Moreno SNJ (1990) The metabolism and mode of action of gentian violet. *Drug Metab Rev* 22: 161–178
- Fairclough R (1963) A comparison of metamidium, Samorin, Berenil and ethidium bromide under field conditions in Kenya. *Vet Rec* 75: 855–858
- Gimbi AA, Kinabo LDB (1992) Influence of atropine on the acute toxicity of isometamidium. *Vet Hum Toxicol* 34: 398–400
- Jones SRM, Woo PTK, Stevenson RMW (1986) Immunosuppression in *Salmo gairdneri* Richardson to the haemoflagellate *Cryptobia salmositica* Katz 1951. *J Fish Dis* 10: 395–402
- Kinabo LDB, Bogan JA (1987) Binding of isometamidium to calf thymus DNA and lipids: pharmacological implications. *J Vet Pharmacol Ther* 10: 357–362
- Kinabo LDB, Bogan JA, McKellar QA, Murray M (1989) Relay bioavailability and toxicity of isometamidium residues: a model for human risk assessment. *Vet Hum Toxicol* 31: 417–421
- Kinabo LDB, McKellar QA, Eckersall PD (1991) Isometamidium: disposition kinetics, tissue residues and adverse reactions. *Res Vet Sci* 50: 6–13
- Kirby WW (1964) A trial to compare the therapeutic activity in cattle trypanosomiasis of homidium and isometamidium. *Bull Epizoot Dis Afr* 11: 299–301
- Li S, Woo PTK (1991) Anorexia reduces the severity of cryptobiosis in *Oncorhynchus mykiss*. *J Parasitol* 77: 467–471
- Li S, Woo PTK (1995) Efficacy of a live *Cryptobia salmositica* vaccine, and the mechanism of protection in vaccinated rainbow trout, *Oncorhynchus mykiss*, against cryptobiosis. *Vet Immunol Immunopathol* 48: 343–353
- Murilla GA, Mdachi RE, Karanja WM (1996) Pharmacokinetics, bioavailability and tissue residues of (14C)isometamidium in non-infected and *Trypanosoma congolense*-infected Boran cattle. *Acta Trop* 61: 277–293
- Philips FS, Sternberg SS, Cronin AP, Sodergren JE, Vidal PM (1967) Physiologic disposition and intracellular localization of isometamidium. *Can Res* 27: 333–349
- Putz RE (1972) Biological studies on the hemoflagellates *Cryptobia cataractae* and *Cryptobia salmositica*. *Tech Pap Bur Sport Fish Wildl* 63: 3–25
- Robson J (1962) Prophylaxis against trypanosomiasis in zebu cattle. IV. A field trial of metamidium and isometamidium. *Vet Rec* 74: 913–917
- Sitja-Bobadilla A, Woo PTK (1994) An enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against the pathogenic hemoflagellate, *Cryptobia salmositica* Katz, and protection against cryptobiosis in juvenile rainbow trout, *Oncorhynchus mykiss* (Walbaum) inoculated with a live vaccine. *J Fish Dis* 17: 399–408
- Sutherland IA, Codjia V, Moloo SK, Holmes PH, Peregrine AS (1992) Therapeutic activity of isometamidium chloride in Boran cattle against a tsetse-transmitted clone of *Trypanosoma congolense* with a low level of drug resistance. *Trop Anim Health Prod* 24: 157–163
- Thomas PT, Woo PTK (1988) *Cryptobia salmositica*, in vitro and in vivo study on the mechanism of anemia in infected rainbow trout, *Salmo gairdneri* Richardson. *J Fish Dis* 11: 425–431
- Thomas PT, Woo PTK (1989a) Complement activity in *Salmo gairdneri* Richardson infected with *Cryptobia salmositica* (Sarcocystidophora: Kinetoplastida) and its relationship to the anaemia in cryptobiosis. *J Fish Dis* 12: 395–397
- Thomas PT, Woo PTK (1989b) An in vitro study on the haemolytic components of *Cryptobia salmositica*. *J Fish Dis* 12: 89–93
- Thomas PT, Woo PTK (1991) In vitro and in vivo effects of antimicrobial agents on viability of *Cryptobia salmositica* (Sarcocystidophora: Kinetoplastida). *Dis Aquat Org* 10: 7–11
- Wehnert SD, Woo PTK (1981) The immune response of *Salmo gairdneri* during *Trypanoplasma salmositica* infection. *Bull Can Soc Zool* 11: 100 (Abstract)
- Weisenhütter E, Turner DB, Kristensen KA (1968) Aspects of current bovine trypanosomiasis control in Tanzania. A comparative field trial of available chemoprophylactics under ranching conditions. *Bull Epizoot Dis Afr* 16: 419–424
- Whitelaw DD, Bell IR, Holmes PH, Moloo SK, Hirumi H, Urquhart GM, Murray M (1986) Isometamidium chloride prophylaxis against *Trypanosoma congolense* challenge and the development of immune responses in Boran cattle. *Vet Rec* 118: 722–726
- Williamson J (1970) Review of chemotherapeutic and chemoprophylactic agents. In: Mulligan HW (ed) *The african trypanosomiasis*. Pitman Press, Bath, pp 125–221
- Woo PTK (1969) The haematocrit centrifuge for the detection of trypanosomes in blood. *Can J Zool* 47: 921–923

- Woo PTK (1979) *Trypanoplasma salmositica*: experimental infections in rainbow trout, *Salmo gairdneri*. *Exp Parasitol* 47: 36–48
- Woo PTK (1992) Immunological responses of fish to parasitic organisms. In: Faisal M, Hetrick FM (eds) *Annual review of fish diseases*, vol 2. Pergamon Press, New York, pp 339–366
- Woo PTK (1994) Flagellate parasites of fishes. In: Krier JP (ed) *Parasitic Protozoa*, vol VIII, 2nd edn. Academic Press, London, pp 1–80
- Woo PTK, Li S (1990) In vitro attenuation of *Cryptobia salmositica* and its use as a live vaccine against cryptobiosis in *Oncorhynchus mykiss*. *J Parasitol* 76: 752–755
- Woo PTK, Poynton SL (1995) Diplomonadida, Kinetoplastida and Amoebida (Phylum Sarcomastigophora). In: Woo PTK (ed) *Fish diseases and disorders I. Protozoan and metazoan infections*. CAB International, Oxford, pp 27–96
- Woo PTK, Thomas PT (1991) Polypeptide and antigen profiles of *Cryptobia salmositica*, *C. bullocki* and *C. catostomi* (Kinetoplastida: Sarcomastigophora) isolated from fishes. *Dis Aquat Org* 11: 201–205